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Oxidation of 1-Tetradecene by *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa strain Sol 20 was grown on 1-tetradecene as sole carbon source, and a vinyl-unsaturated 14-carbon monocarboxylic acid, 13-tetradecenoic acid, was identified from culture fluid. This acid was not produced when *n*-tetradecane served as substrate for growth. Oxidation of the methyl group represents one method of attack on the 1-alkene by this organism. Tentative identification of 2-tetradecanol indicates that an attack on the double bond is also occurring. α,ω-Dienes would not support growth.

McKenna and Kallio (10), in a recent review on the biology of hydrocarbons, noted that, whereas unsaturated aliphatic hydrocarbons serve as substrates for various microorganisms, the pathways concerned with alkene degradation are not as well defined as those for alkanes.

Bruyn (2) demonstrated that *Candida lipolytica* produced the corresponding 1,2-diol from 1-hexadecene during growth on the alkene in a defined medium. This observation was subsequently confirmed for this yeast (13), but diol formation from alkenes mediated by bacteria has not been observed. The recovery of 15-hexadecenyl palmitate from cultures of *Micrococcus cerificans* growing on 1-hexadecene (13), and the identification of 6-heptenoic acid as an oxidation product of 1-heptene by heptane-grown cells of a *Pseudomonas* strain (14), indicate that alkenes are subject to oxidation at the saturated end of the molecule.

Van der Linden (15) and Huybregtse and van der Linden (5) extended the study of alkene oxidation by *P. aeruginosa*, and concluded that minor reactions occur at the unsaturated end of the alkene, leading to the formation of an epoxide and a saturated acid (possibly via the aldehyde). Saturated methyl ketones or saturated alcohols were not detected. Methyl group oxidation was considered to be the main degradative pathway for alkene oxidation.

Senez and Azoulay (12) and Azoulay, Chouteau, and Davidovics (1), using strain Sol 20 of *P. aeruginosa*, concluded that alkanes are degraded through an alkene intermediate that undergoes epoxidation followed by reduction to a primary alcohol which subsequently is oxidized to an acid. This implies that alkanes and alkenes are degraded by a single pathway and that the oxidative products of both hydrocarbons should be the same.

This report concerns the oxidation of a long-chain alkene, 1-tetradecene, by *P. aeruginosa* strain Sol 20.

MATERIALS AND METHODS

Organism and culture methods. A strain of *P. aeruginosa*, Sol 20, was obtained from J. C. Senez, Marseille, France. Large-scale cultures were grown in 2.8-liter Fernbach flasks containing 1 liter of the mineral medium of Dworkin and Foster (4) at *p*H 7. Inoculum was 1% of 4-to 5-day growth of the organism on the same medium. Filter-sterilized 1-tetradecene at a concentration of 0.5% (v/v) was added at the time of inoculation. Cultures were grown without agitation for 4 to 5 days at 30 C.

1-Tetradecene with a 99 mole per cent purity was obtained from the Humphrey Co., North Haven, Conn. Oxygenated impurities were removed from the olefin by passing it through a column of Adsorbosil-S2 (Applied Science Laboratories, State College, Pa.). Fractions of the olefin that were free from oxygenated compounds by thin-layer and gas-liquid chromatographic criteria were used as substrate.

Recovery and detection of intermediates. Culture fluids, freed from cells, were acidified to *p*H 1 with H_2SO_4 and extracted continuously with diethyl ether for 48 hr. Ether volume was reduced, and the sample was added to a column of Silica Gel H. Residual olefin was removed by elution with hexane. Fractionation was accomplished by elution with *n*-hexane-diethyl ether-acetic acid (80:20:1, v/v). Collected fractions were assayed by thin-layer chromatography on Adsorbosil-1 with hexane-diethyl ether-acetic acid as developing solvent. Spots were detected by spraying the plates with a 0.2% ethanolic solution of 2',7'-dichlorofluorescein and were visualized under ultraviolet light. Fractions showing spots corresponding to monocarboxylic acid and to monohydroxy alcohol were pooled separately.

Preparative thin-layer chromatography was used in some cases to recover acids and alcohols from ether extracts of fluid. Adsorbosil-1 plates (20 by 20 cm), 250 μ thick, were spotted, developed in *n*-hexane to move unused olefin to the top of the plate, and then redeveloped in *n*-hexane-diethyl ether-acetic acid (80:20:1, v/v). Positions of the spots were recognized by staining only the edges of the plate with 2',7'-dichlorofluorescein. Silica gel from areas of the plates corresponding to monocarboxylic acids and monohydroxy alcohols was removed, and compounds were extracted from the gel with chloroform.

Analyses of the alcohol and acid fractions were obtained by gas-liquid chromatography. Analysis of alcohols was obtained on a column of 2% Versamid 900 on Chromosorb AW-DMCS 60–80 mesh packed in a 6-ft (1.8-meter) copper column with an internal diameter of ⅛ inch (0.3 cm) and on a 6-foot copper column (⅛ inch internal diameter) containing column packing described elsewhere (8). Methyl esters of fatty acids were identified on two columns: (i) 10% diethylene glycol adipate (Lac-446) on Diatoport 60/80 mesh in an 8-ft (2.4-meter) copper column with an internal diameter of ¼ inch (0.6 cm), (ii) 10% Apiezon L on Chromosorb W 45/60 mesh packed in a 7-ft (2.1-meter) copper column (¼ inch internal diameter). Columns of ⅛ inch diameter were used in an Aerograph HyFi model 600 chromatograph and columns ¼ inch in diameter were used in an F and M model 700 chromatograph. Both instruments were equipped with hydrogen flame detectors.

Methyl esters of fatty acids were identified by comparing retention times of extracted compounds with synthesized compounds and known standards obtained from Applied Science Laboratories. Alcohols used as standards for gas-chromatographic comparisons were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Preparative gas-liquid chromatography was used for the recovery of the 14-carbon acids present in the monocarboxylic acid fractions obtained from preparative thin-layer and column chromatography. Free fatty acids were collected from a column reported previously (8). Separation of saturated and unsaturated acids of the same carbon chain length was accomplished by adsorption chromatography on silver nitrate-impregnated silica gel (Adsorbosil-ADN-1, Applied Science Laboratories) by the method of De Vries (3). Better recovery of individual acids was obtained by preparative gas-liquid chromatography of the methyl esters on a column of 10% diethylene glycol adipate (Lac-2R-446) on Chromosorb W 45/60 mesh packed in a 7-ft copper column with an internal diameter of ¼ inch. Helium was the carrier gas employed at a flow rate of 140 ml/min; column temperature was 155 C. The preparative gas chromatograph was an Autoprep model A-700 (Varian-Aerograph, Walnut Creek, Calif.).

Chemical. 1,7-Octadiene was obtained from Columbian Carbon Co., Princeton, N.J. 13-Tetradecenoic acid and 15-hexadecenoic acid were synthesized by the Department of Chemistry, University of Iowa. 1,13-Tetradecadiene was synthesized by reacting 11-bromo-1-undecane with magnesium followed by allyl bromide (9). Methyl esters of fatty acids were prepared and purified on a Florisil column (11). The presence of unsaturated compounds was determined by peak disappearance in gas-liquid chromatographic analyses after bromination by the procedure of James (6). Infrared spectra were obtained with a Perkin-Elmer Model 137B spectrophotometer with the use of NaCl cavity cells with a path length of 0.1 mm.

RESULTS

Acids. The thin-layer chromatographic indication of a monocarboxylic acid class of compounds present in fluid of cultures grown on 1-tetradecene led to the speculation that these compounds would be predominantly acids of 14 or fewer carbon atoms, i.e., degradative products of the olefin. However, gas-liquid chromatographic analyses indicated a broad spectrum of acids with less than, and greater than, 14 carbons.

Figure 1 represents portions of gas-liquid chromatographic scans of methyl esters. Certain

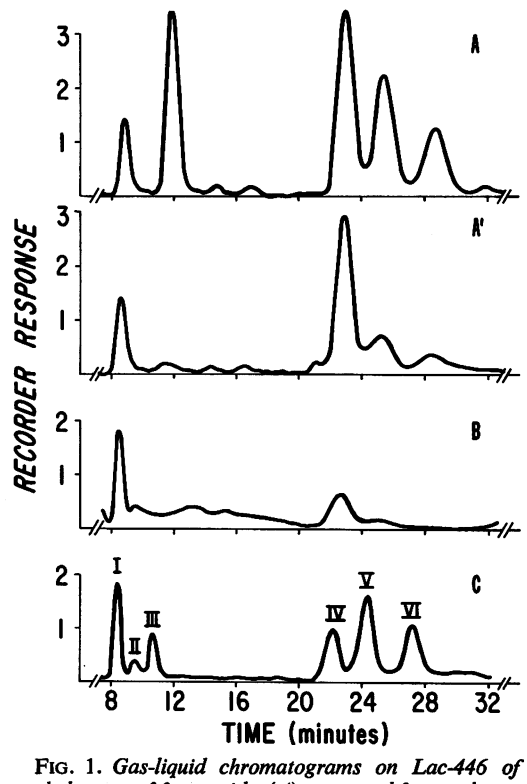

FIG. 1. *Gas-liquid chromatograms on Lac-446 of methyl esters of fatty acids: (A) extracted from cultures grown on 1-tetradecene; (A') same as (A) except after bromination; (B) extracted from cultures grown on n-tetradecane; (C) methyl ester standards of (I) tetradecanoic acid, (II) Δ⁹-tetradecenoic acid, (III) 13-tetradecenoic acid, (IV) hexadecanoic acid, (V) Δ⁹-hexadecenoic acid, (VI) 15-hexadecenoic acid.*

peaks were significantly reduced after bromination, thereby indicating unsaturation. Part A is a scan, before and after bromination, of acids extracted from fluid of a culture grown on 1-tetradecene; part B shows a scan of acids extracted from a culture grown on *n*-tetradecane. Peaks corresponding to standards III and VI do not appear when *n*-tetradecane served as growth substrate. Part C is a scan of methyl esters of known standards: (I) tetradecanoic acid, (II) Δ^9-tetradecenoic acid, (III) 13-tetradecenoic acid, (IV) hexadecanoic acid, (V) Δ^9-hexadecenoic acid, (VI) 15-hexadecenoic acid. Saturated acids of 12 and 13 carbons were also seen from 1-tetradecene cultures, but no unsaturated peaks corresponding to these chain lengths were detected. The unsaturated 14-carbon peak was usually present in a greater concentration than the saturated-acid peak. Δ^9-Tetradecenoic acid could be detected in trace amounts in some analyses from both *n*-tetradecane and 1-tetradecene cultures.

Figure 2 illustrates an infrared spectral analysis of the 14-carbon unsaturated acid. Absorption bands at 1,650, 990, and 910 cm^{-1} are indicative of a terminal vinyl double bond which is present in 13-tetradecenoic acid. Spectra of the isolated acid and the synthesized 13-tetradecenoic acid compare favorably.

Mass spectral analysis of the methyl ester of the terminally unsaturated acid showed the major fragment to have a molecular weight of 240. From gas-liquid chromatographic, infrared, and mass spectral analyses, the unsaturated 14-carbon acid appears to be 13-tetradecenoic acid.

Alcohols. A class of compounds corresponding to alcohols was recovered by preparative thin-layer chromatography and analyzed by gas-liquid chromatography on two different columns. One peak gave retention times on both columns corresponding to authentic 2-tetradecanol. Bromination did not reduce peak area, and the addition of authentic 2-tetradecanol to the sample increased the height of the unknown peak (Fig. 3). No tentative assignments have been given to the other peaks represented in Fig. 3.

Diene utilization. 1,7-Octadiene and 1,13-tetradecadiene were added at concentrations of 1.0 and 0.5% as the sole carbon source in growth experiments. No growth occurred after 2 weeks in either agitated or nonagitated flasks. However, organisms tentatively identified as *Pseudomonas* species have been obtained by enrichment culturing with these dienes at a concentration of 1.0%.

DISCUSSION

The presence of 13-tetradecenoic acid in cultures grown on 1-tetradecene indicates a direct attack on the methyl group of the alkene. A two-

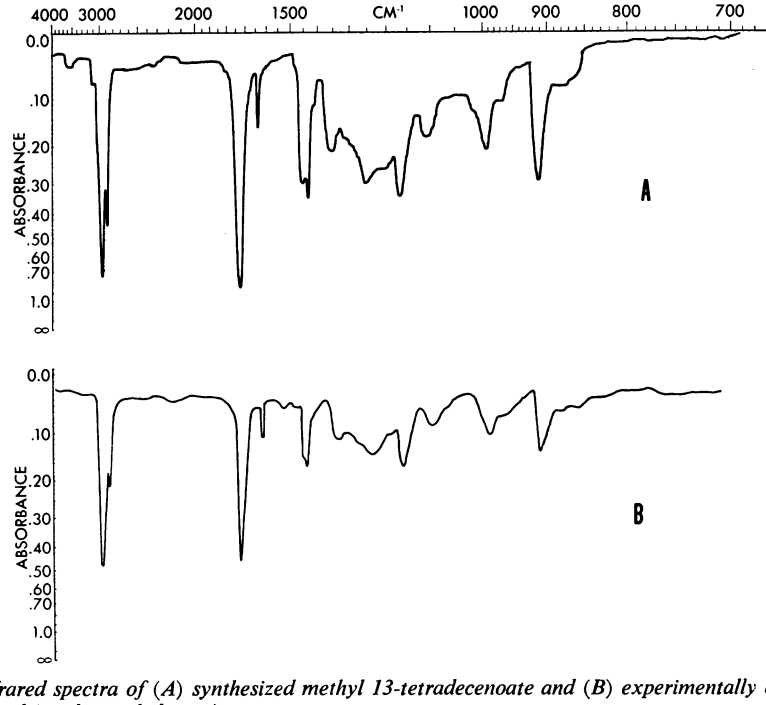

FIG. 2. *Infrared spectra of (A) synthesized methyl 13-tetradecenoate and (B) experimentally derived 14-carbon unsaturated acid (as the methyl ester).*

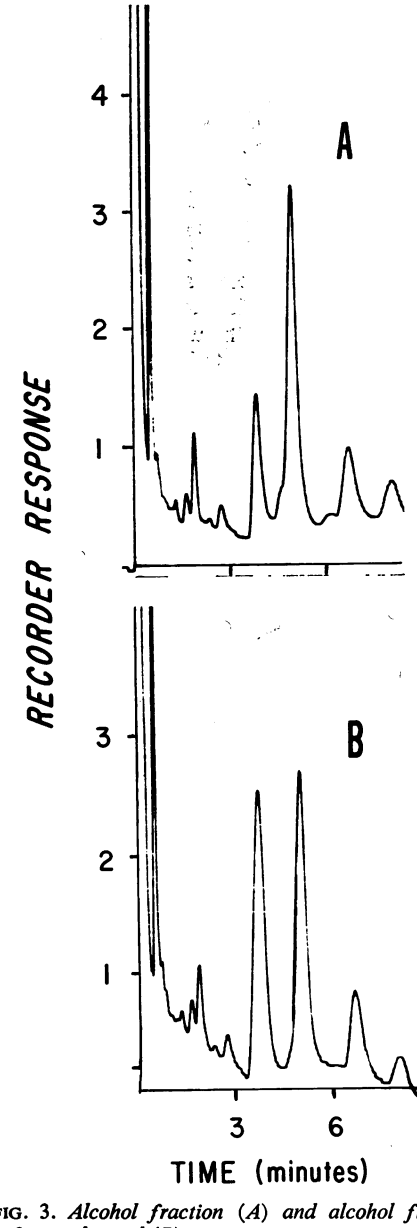

FIG. 3. *Alcohol fraction (A) and alcohol fraction plus 2-tetradecanol (B).*

carbon addition to this ω-unsaturated acid would account for the presence of 15-hexadecenoic acid.

The ratio of peak areas of tetradecanoic acid and 13-tetradecenoic acid is usually in favor of the terminally unsaturated acid. Huybregtse and van der Linden (5) observed this relationship in their analysis of 7-octenoic and octanoic acids from the oxidation of 1-octene by a *Pseudomonas* species, and this was interpreted as evidence for the major degradation of the α-olefin via the ω-unsaturated fatty acid. On the basis of the data presented here, it would be presumptive to assign a predominant degradative pathway to the α-olefin via the ω-unsaturated acid, based solely on a concentration ratio relative to the saturated acid. The possibility exists that 13-tetradecenoic acid is formed by a degradative pathway of minor importance, and that it accumulates because it is degraded slowly or not at all. In support of this speculation, unsaturated 12- or 13-carbon acids, which would be expected as degradative products from the unsaturated 14-carbon acid, were not detected.

Gas-liquid chromatographic identification of 15-hexadecenoic acid does, however, indicate that the ω-unsaturated 14-carbon acid can participate in some biological reactions mediated by *P. aeruginosa* (Sol 20), i.e., chain elongation by two-carbon addition.

Whatever the relative importance of the ω-unsaturated acid, its presence is not in accord with the products expected if this organism oxidizes alkanes through an alkene intermediate, as reported by Senez and Azoulay (12) and Azoulay, Chouteau, and Davidovics (1). This pathway gives rise to a saturated primary alcohol by a purported epoxidation of the double bond and subsequent reduction to a saturated alcohol followed by oxidation to the acid.

It may be that the 14-carbon saturated acid, also isolated in the present study, is not entirely a product resynthesized from the carbon obtained by the degradation of 1-tetradecene, serving as sole carbon source; alternatively, this acid may arise in part from some direct alteration of the double bond of the alkene. Tentative identification of 2-tetradecanol indicates that an attack on the double bond of the substrate is occurring, but this intermediate would not serve as a precursor for a 14-carbon saturated acid.

Knipprath and Mead (7) fed 15-hexadecenoic acid, ^{14}C-labeled in the carboxyl group, to rats. Based on the amount of label found in palmitic acid, these authors suggested the possibility that the terminal double bond of the unsaturated acid was hydrogenated. A reaction of this type, although unlikely, could be operative in the pseudomonad system; this would give rise to a saturated 14-carbon acid from 13-tetradecenoic acid.

The relative importance of catabolic reactions involving the olefinic double bond remains uncertain. However, the inability of *P. aeruginosa* (Sol 20) to grow on α,ω-unsaturated dienes casts doubt on alteration of the double bond as an important step in the major pathway by which olefins are oxidized by this organism.

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